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16. ABSTRACT

Rat embryos removed from the dam on day 10 of pregnancy were successfully co-cultivated in vitro with primary cultures of rat, rabbit, or hamster hepatocytes. Embryos co-cultivated with hepatocytes developed normally, as did embryos exposed to a test chemical, cyclophosphamide. In a system where embryos were co-cultivated with hepatocytes and then exposed to cyclophosphamide a dose-related embryotoxicity was observed, indicating metabolic activation of the proteratogen. When similar numbers of hepatocytes were used for metabolic activation of cyclophosphamide, the induced embryotoxicity was greater in the presence of rabbit and hamster hepatocytes than with rat hepatocytes.

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Co-Culture of Rat Embryos and Hepatocytes: In Vitro Detection of a Proteratogen

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The technique of whole embryo culture developed by New [Environ Health Perspect 18:105–110, 1976] provides a sensitive assay to evaluate the effects of a test chemical on embryo development independent of maternal influences. To detect proteratogens, this assay must be coupled with an exogenous metabolic activation system. We have developed methods for the co-cultivation of rat embryos with primary hepatocytes, which offers several advantages over subcellular fractions when providing metabolic activation for in vitro assays. In the present study, rat embryos removed from the dam on day 10 of pregnancy were co-cultivated in vitro with primary cultures of rat, rabbit, or hamster hepatocytes. Embryos co-cultivated with hepatocytes developed normally, as did embryos exposed to a test chemical, cyclophosphamide (CP) in the absence of hepatocytes. When embryos were co-cultivated with hepatocytes and exposed to CP, a dose-related embryotoxicity was observed, indicating metabolic activation of the proteratogen. Using hepatocytes isolated from rats pretreated in vivo with phenobarbital, we observed an increase in CP-induced malformations and embryotoxicity compared to those of embryos exposed to CP in the presence of uninduced hepatocytes. The teratogenic bioactivation of CP was inhibited in vitro by the addition of metyrapone. When similar numbers of hepatocytes were used for metabolic activation of CP the induced embryotoxicity was greater in the presence of rabbit and hamster hepatocytes than with rat hepatocytes. Development of procedures for the culture of rat embryos with hepatocytes from other species

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suggests the utility of this in vitro system for the investigation of species differences in sensitivity to chemical teratogens.

Key words: teratogenicity, embryotoxicity, cyclophosphamide, bioactivation, whole embryo culture, co-cultivation

INTRODUCTION

The co-cultivation of rodent embryos and hepatocytes from diverse species offers the opportunity to observe mammalian development in environments containing unique arrays of species-specific metabolites. Such a system may enable investigators to study the role(s) of maternal metabolism in the production of birth defects as well as ultimately allowing the observation of rodent development in a milieu containing human hepatocytes. This paper reports on the development of such a co-cultivation system.

The technique of whole embryo culture developed by New [1] is being used increasingly both as a proposed teratology screening assay and in the study of basic mechanisms involved in the production of birth defects. As a screening assay it offers the advantage of being less expensive and quicker. In the study of mechanisms, advantages include precise control of dosage and stage of development at the time of exposure. Also, it is possible to evaluate the effects of the test chemical on the embryo independent of maternal influences. In order to examine teratogens that may require bioactivation to exert their effects, an exogenous metabolic activation system, either a 9,000g (S9) supernatant or a purified microsomal fraction from homogenized liver, has been added to embryo cultures [2,3]. Liver cell homogenates may not be a good model for in vivo metabolism because the preparation procedure destroys subcellular structure and compartmentalization, which results in the loss of sequence of various metabolic reactions and does not provide the full complement of enzymes and cofactors present in intact hepatocytes. In fact, subcellular fractions have been shown to differ significantly from intact cells in patterns of metabolite profiles [4,5]. Another disadvantage is the nonspecific cytotoxicity as well as embryotoxicity of microsomal fractions in many in vitro assays [6–8].

For similar reasons, cellular metabolic activation systems have been coupled to a variety of in vitro assays for genotoxicity [9].

The potential benefits of adding intact cells to embryo cultures have been recognized for some time, but the significant differences in culture requirements for embryos and hepatocytes have been a barrier to the development of a co-cultivated system. Rodent embryos are traditionally cultured in vitro in roller culture at 30 rpm with rat serum as the culture medium. The culture tubes are sealed except for periodic gassing with 5% CO_2 and increasing concentrations of O_2 [10]. In comparison, hepatocytes are generally prepared in stationary culture in one of several commercially available cell culture media supplemented with 1–20% fetal bovine serum. The cells are seeded into loosely capped culture flasks and maintained in continuous equilibrium with a 5% CO_2 in air atmosphere maintained within an incubator [11]. One approach to co-cultivation has been to add hepatocytes in suspension directly to embryo culture [12], but the prolonged viability of hepatocytes in monolayer culture along with evidence that freshly isolated hepatocytes have impaired functions that are

repaired during primary culture [13] make the use of hepatocyte monolayer cultures for metabolic activation desirable.

Here we report the development of procedures for the co-cultivation of rat embryos and hepatocytes. Also, to validate the ability of intact hepatocytes to serve as a metabolic activation system for proteratogens in vitro, CP was selected as a test chemical because it is a well-characterized teratogen known to require maternal metabolic activation [14].

MATERIALS AND METHODS

Embryo Culture

Nulliparous female Sprague Dawley rats obtained from Charles River were time-mated on site in our animal facility. Vaginal smears were made on the morning following the mating and the presence of sperm confirmed pregnancy (day 0 of pregnancy). Embryos were explanted from the rats in the afternoon of day 10 and maintained in roller culture in immediately centrifuged, heat-inactivated male rat serum according to the procedures of New [1] during hepatocyte attachment (see Fig. 1).

Hepatocyte Isolation and Culture

Hepatocytes were isolated from adult male Sprague Dawley rats (Charles River), New Zealand white rabbits (Dutchland Laboratory Animals, Inc.), and Syrian golden hamsters (Harlan Sprague Dawley, Inc.) by the slicing technique of Fry et al [15]. Cell viability was determined by trypan blue dye exclusion and only preparations with viability greater than 80% were used. Hepatocytes were seeded into 25-cm^2 tissue culture flasks that had been precoated with fibronectin (Collaborative Research) at 2 μg fibronectin/cm^2 of culture substrate. Hepatocytes were seeded at a density of 3×10^6 viable cells per flask in Waymouth's MB 752/1 medium supplemented with 9 mM sodium bicarbonate, 5 mM Hepes buffer, and 2 mM L-glutamine (Gibco). The

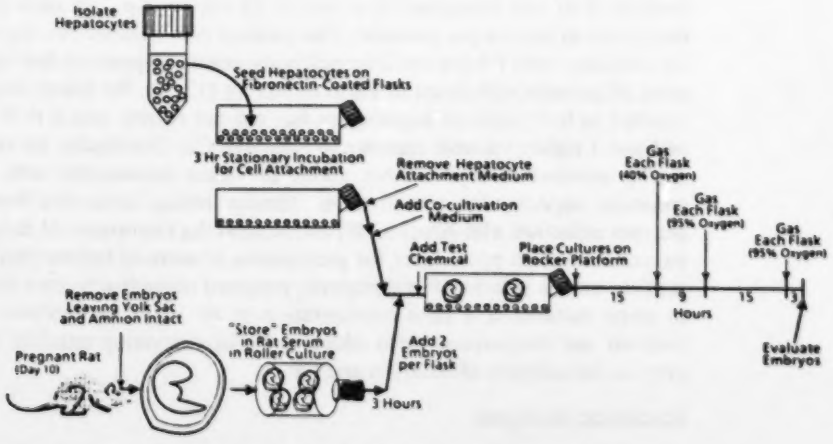

Fig. 1. Procedure for the in vitro embryo/hepatocyte teratogenesis assay.

medium was also supplemented with 17% fetal bovine serum for rat and rabbit hepatocytes and 1% for hamster hepatocytes [11]. At the end of a 3 h cell attachment period, 1.6 to 2.0 × 10^6 hepatocytes were attached per 25 cm^2 flask. The number of hepatocytes attached per flask was determined by counting the number of cells in several fields of view on an inverted microscope fitted with an eyepiece reticule. A similar number of hepatocytes attached to each flask regardless of the species from which they were isolated.

Co-cultivation of Embryos and Hepatocytes

When the hepatocytes were attached, the culture medium and any unattached hepatocytes were removed from the flasks and 10 ml of co-cultivation medium and two embryos were added to each flask (see Fig. 1). The co-cultivation medium was composed of 50% Waymouth's MB 752/1 medium, 35% heat-inactivated male rat serum, 15% heat-inactivated fetal bovine serum, and also contained penicillin (50 units/ml) and streptomycin (50 μg/ml). The medium was gassed with a mixture of 40% O_2, 5% CO_2 and 55% N_2 for 30 min. At the initiation of co-cultivation, the flasks were placed on a rocker platform at 32 oscillations/min at 37°C for 42 h. The flasks were gassed with a mixture of 40% O_2, 5% CO_2, and 55% N2 after 15 h in culture and with a mixture of 95% O_2 and 5% CO_2 after 24 and 39 h.

Assay for Embryotoxicity

A 1-mg/ml stock solution of CP (Sigma) was prepared in Hanks' balanced salt solution immediately prior to each experiment and CP was added at the initiation of co-cultivation (Fig. 1). After 42 h in culture, viable embryos were evaluated for growth (crown-rump length and somite differentiation) and examined for morphological abnormalities. Embryos were sonically homogenized and the total DNA determined by the method of Boer [16].

In order to further characterize co-cultivated hepatocytes as a bioactivation system for proteratogens, we studied the effect of phenobarbital, an inducer of hepatic cytochrome P-450, and metyrapone, a known inhibitor of cytochrome P-450. Phenobarbital (PB) was administered to rats at 40 mg/kg, i.p., on each of 4 consecutive days prior to hepatocyte isolation. This method is in contrast to a common procedure for induction with PB prior to liver cell homogenate preparation that includes pretreatment of animals with doses of PB at 80 mg/kg [17,18]. We found that this procedure resulted in low yields of hepatocytes that did not readily attach to the culture flasks and had a highly variable capacity to bioactive CP. Decreasing the dose of PB to 40 mg/kg resulted in reproducible yields of viable hepatocytes with a significantly enhanced capacity for bioactivation. Similar results have also been reported for enzyme induction with Aroclor [19] and suggest the importance of modifying standard enzyme induction procedures for preparation of induced hepatocytes. For inhibition studies, a stock solution of metyrapone, prepared immediately prior to use, was added to some cultures at a final concentration of 40 μg/ml of medium. Co-cultivation medium and metyrapone were added to flasks containing attached hepatocytes 1 h prior to the addition of embryos and CP.

Statistical Analysis

For the straight co-cultivation experiments, somite number and embryonic DNA concentration data were analyzed by ANOVA procedures followed by pairwise

comparisons of the least square means using Student's t-test when the ANOVA was significant. In the induction and inhibition experiments, these data were analyzed by a two-way ANOVA (metabolic modifier and dose) with interactions followed by pairwise comparisons. A chi-square test was used for analysis of the malformation data. A probability of main effect or interaction of $p < 0.05$ was considered statistically significant.

RESULTS

The procedures detailed in Figure 1 supported the successful co-cultivation of day 10 rat embryos and primary cultures of rat hepatocytes. Embryos cultured for 42 h in the presence of hepatocytes developed normally (Table I). Embryos exposed to CP in the absence of rat hepatocytes also developed normally while exposure to CP in the presence of hepatocytes resulted in a concentration-related decrease in somite number and total embryonic DNA. With approximately 2×10^6 hepatocytes as the exogenous metabolic activation system as little as 6.25 μg/ml CP caused a significant decrease in embryo DNA in co-cultivated cultures. This level was not toxic to the hepatocytes based on viability determinations by trypan blue dye exclusion following exposure (dose levels up to 25 μg/ml had no significant effect). A concentration-related increase in incidence of abnormal embryos was observed following exposure to CP in the presence of hepatocytes. The number of malformations observed per embryo also increased with increasing doses of CP. Characteristic embryo abnormalities [2,3] included missing limb buds, edematous blisters, shortened tails, and ventrolateral protrusions adjacent to the prosencephalon.

Cyclophosphamide embryotoxicity and teratogenicity was significantly enhanced if hepatocytes were isolated from rats pretreated with phenobarbital (PB). In the presence of PB-induced hepatocytes, exposure to 3.0 μg/ml CP resulted in a 79% incidence of malformations (Table II). At 6.5 μg/ml there was significant embryotoxicity and 100% malformations, while in comparison the same dose in the presence of control hepatocytes caused only a slight effect. To produce a similar amount of CP-induced damage, approximately a fourfold higher concentration of CP was required in the presence of control hepatocytes compared to bioactivation by PB-induced hepatocytes.

Addition of metyrapone, an inhibitor of cytochrome P-450-dependent monooxygenase activity, was effective in reducing CP-induced embryotoxicity and terato-

TABLE I. Growth and Development of Rat Embryos Exposed to Cyclophosphamide (CP) In Vitro

Hepatocytes	CP (μg/ml)	No. of embryos	Somites	DNA content (μg/embryo)	Percentage abnormal
−	−	9	35.7 ± 0.6[a]	42.4 ± 2.5	11
+	−	38	34.8 ± 0.3	47.4 ± 2.4	5
−	25.00	25	33.8 ± 0.4	41.6 ± 2.2	13
+	3.00	17	34.4 ± 0.4	41.5 ± 3.1	0
+	6.25	31	33.8 ± 0.5	37.2 ± 3.0[b]	28[b]
+	12.50	48	31.1 ± 0.4[b]	26.4 ± 1.5[b]	60[b]
+	18.75	20	30.1 ± 0.7[b]	22.3 ± 1.7[b]	95[b]
+	25.00	16	30.5 ± 0.7[b]	22.2 ± 2.3[b]	88[b]

[a]Values shown are mean ± SE

[b]$p < 0.05$ compared to nontreated control.

TABLE II. Enhancement of cyclophosphamide-induced teratogenicity In Vitro*

	Cyclophosphamide (μg/ml)		
	0	3.0	6.25
Somites			
Control	36.8 ± 0.3[a]	36.8 ± 0.3	35.8 ± 1.1
PB-induced	37.4 ± 0.3	35.1 ± 0.7[c]	28.2 ± 0.8[c,d]
Crown-rump length (mm)			
Control	5.2 ± 0.1	5.1 ± 0.1	4.8 ± 0.2[b]
PB-induced	5.2 ± 0.1	4.7 ± 0.1[c,d]	4.0 ± 0.1[c,d]
DNA Content (μg/embryo)			
Control	76.3 ± 4.4	66.5 ± 3.5	53.1 ± 5.1[b]
PB-induced	78.2 ± 5.1	46.8 ± 4.0[c,d]	27.0 ± 1.8[c,d]
Abnormal/alive			
Control	0/16	0/15	2/16
PB-induced	2/16	11/14[b,c,d]	12/12[b,c,d]

*Phenobarbital (PB) was administered to rats at 40 mg/kg, i.p., on each of 4 consecutive days prior to hepatocyte isolation.
[a]Values shown are mean ± SE
[b]$p < 0.05$ compared to uninduced control.
[c]$p < 0.05$ compared to induced control.
[d]$p < 0.05$ compared to matching uninduced group.

TABLE III. Inhibition of Cyclophosphamide-Induced Teratogenicity In Vitro*

	Cyclophosphamide (μg/ml)	
	0	25
Somites		
Control	36.3 ± 0.3	29.2 ± 1.0[a,b,c]
Metyrapone	35.8 ± 0.2	35.8 ± 0.3
Crown-rump length (mm)		
Control	5.2 ± 0.1	4.2 ± 0.1[a,b,c]
Metyrapone	5.1 ± 0.1	4.9 ± 0.1[a]
DNA content (μg/embryo)		
Control	61.8 ± 4.6	22.7 ± 2.0[a,b,c]
Metyrapone	60.7 ± 3.0	55.6 ± 4.0
Abnormal/alive		
Control	1/18	13/13[a,b]
Metyrapone	3/18	9/18[c]

*Metyrapone at 40 μg/ml of co-cultivation medium was added 1 h before the addition of embryos and cyclophosphamide; values shown are mean ± S.E.
[a]$p < 0.05$ compared to noninhibited control.
[b]$p < 0.05$ compared to inhibited control.
[c]$p < 0.05$ compared to matching CP-exposed inhibited group.

genicity. In the presence of hepatocytes, 25 μg/ml CP caused a significant decrease in growth and resulted in 100% malformations. When 40 μg/ml metyrapone was added to this system, embryo somite number was not significantly different from concurrent controls grown either in medium alone or medium plus metyrapone, crown-rump length only slightly decreased, and the incidence of malformations was reduced to 50% (Table III).

Day 10 rat embryos can also be successfully co-cultivated with hepatocytes from rabbit or hamster. When similar numbers of hepatocytes were used for metabolic activation of CP, the decrease in embryo DNA content was greater in the presence of

rabbit and hamster hepatocytes than with rat hepatocytes (Fig. 2A). Similar CP-induced malformations were observed in the rat embryos regardless of the species of hepatocyte used. The incidence of abnormal embryos at a specific concentration of CP varied with the source of hepatocytes used for metabolic activation (Fig. 2B). Exposure to 6.25 μg/ml CP for 42 h resulted in an incidence of 25% abnormal embryos in the presence of rat hepatocytes, 64% in the presence of rabbit hepatocytes, and 100% abnormal embryos in the presence of hamster hepatocytes. Embryos exposed to either 6.25 μg/ml CP in the presence of hamster hepatocytes or 18.75 μg/ml CP in the presence of rat hepatocytes exhibited a similar decrease in total embryo DNA and number of somites and a similar increase in incidence and types of embryo malformations.

DISCUSSION

We have demonstrated that rat embryos can be co-cultivated with monolayers of intact primary hepatocytes with no detrimental effect on embryo development and

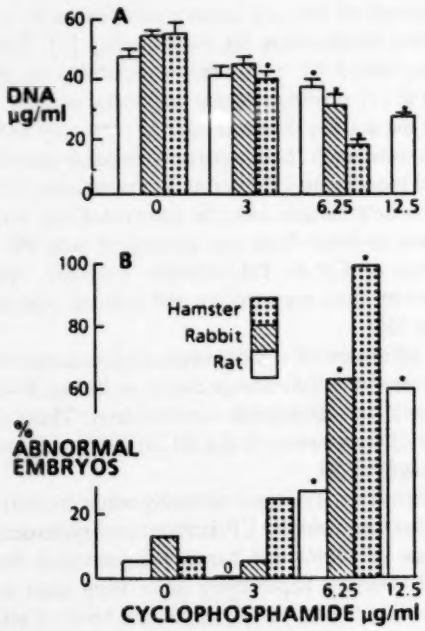

Fig. 2. (A) Effect of hepatocyte source on rat embryo DNA content. Rat embryos were exposed to CP in vitro for 42 h in the presence of rat, rabbit, or hamster hepatocytes as the source of exogenous metabolic activation. Control embryos were co-cultivated with hepatocytes from each species without CP. *, significantly different from control at $p < 0.05$ by Student's t test. (B) Effect of hepatocyte source on incidence of abnormal embryos. Embryos were co-cultivated for 42 h from day 10 with hepatocytes isolated from adult male rats, rabbits, or hamsters in the presence of CP. At the end of the culture period, viable embryos with heart beat and yolk sac circulation were examined for malformations. An asterisk denotes $p < 0.05$ significance from control.

that the hepatocytes can function as a bioactivation system for a proteratogen. Exposure to a proteratogen, CP, produced embryotoxicity and malformations in rat embryos co-cultivated with hepatocytes while embryos exposed to CP in the absence of hepatocytes developed normally. These in vitro embryotoxic and dysmorphogenic effects are similar to those observed in rats exposed in vivo [14].

Direct comparisons with other in vitro systems are difficult to make since liver homogenates prepared from induced animals have generally been used as a source of exogenous bioactivation for proteratogens in vitro and the length of embryo culture varies between 24 and 48 h in different laboratories. Activation of CP by hepatocytes isolated from uninduced rats (Table I) is effective in a concentration similar to that used in studies conducted by Fantel et al [2] with a liver homogenate (S9) prepared from aroclor-induced rats. The concentration of S9 used in that study (75 μl of S9 containing 29 mg protein/ml added per culture bottle), is approximately comparable to 6×10^6 hepatocytes based on the finding that S9 prepared from cells yields an average of 1.3 mg protein per 4×10^6 cells [20]. Since we observed significant CP-induced embryotoxicity in the presence of 2×10^6 hepatocytes, intact hepatocytes appear to be a more efficient bioactivation system for CP. An enhanced capacity to bioactivate CP was also observed with induced hepatocytes. In order to produce approximately a 75% incidence of malformations, a dose of 3 μg/ml CP was required in the presence of induced hepatocytes (Table II) compared to 12.5 μg/ml CP with induced liver homogenate for bioactivation [2]. This difference in sensitivity to CP may be explained by a difference in lability of the metabolic activation system. Kitchin et al [3] investigated the enzymatic activity of microsomes in rat serum and found that the activity dropped rapidly, with only 10% of the initial activity detectable after a 2-h incubation. In comparison, hepatocytes were reported to have a significant capacity to bioactivate CP after an 8-h incubation [17].

Cyclophosphamide-induced embryotoxicity was significantly enhanced if hepatocytes were isolated from rats pretreated with PB. This finding is consistent with bioactivation of CP by PB-inducible P-450(s). Also, these results are similar to embryotoxicity data reported for PB-induced bioactivation of CP both in vitro [18] and in vivo [14].

The inhibition of cyclophosphamide-induced embryotoxicity by metyrapone is also consistent with the concept that cytochrome P-450 is involved in the metabolism of CP to toxic and teratogenic metabolite(s). These data are in agreement with the in vitro study of Greenaway et al [18], in which metyrapone inhibited the bioactivation of CP by rat liver S9.

Rat embryos developed normally when co-cultivated with hepatocytes from rat, rabbit, or hamster, but the CP-induced embryotoxicity was observed to be greater in the presence of rabbit and hamster hepatocytes than with rat hepatocytes. When cultures of primary hepatocytes have been used as a model for in vivo hepatic metabolism in other in vitro systems, the level of activation has varied depending on the source of hepatocytes. Metabolic activation by hamster and rabbit hepatocytes has been demonstrated to be more efficient than rat hepatocytes in other in vitro assays [21,22] and there is some evidence for species-specific production of metabolites in vitro [23,24]. Although there are no studies investigating the relative teratogenicity of CP in rats, rabbits, and hamsters, the teratogenicity and embryotoxicity of CP has been demonstrated in these species [14,25,26].

CONCLUSIONS

We have developed a method for co-cultivation of rat embryos and hepatocytes isolated from rat, rabbit, or hamster that causes no detrimental effect on embryo development. We have also demonstrated that the co-cultivated hepatocytes can serve as an exogenous metabolic activation system for a proteratogen (CP) in vitro. Exposure to CP in the presence of hepatocytes produced embryotoxic and dysmorphogenic effects similar to those observed in rats exposed in vivo [14]. Also, the bioactivation of CP can be modified by a P-450 inducer or inhibitor. Since the system can be constructed with rat embryos and hepatocytes from diverse species, it may provide a useful tool for investigating the mechanisms underlying species differences in sensitivity to chemical teratogens. The addition of human hepatocytes for metabolic activation is an exciting prospect that could make this in vitro system a useful model for human risk assessment.

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